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(54) Title: NOVEL VACCINE COMPOSITIONS FOR HERPES SIMPLEX VIRUS

(57) Abstract

The present invention discloses new vaccine compositions for Herpes Simplex comprising a whole live HSV-2 virus having various amino acids deleted. Methods of using the vaccine compositions are also included.

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TITLE OF THE INVENTION
NOVEL VACCINE COMPOSITIONS FOR HERPES SIMPLEX VIRUS

BACKGROUND OF THE INVENTION

The present invention discloses new vaccine compositions for Herpes Simplex comprising a whole live HSV-2 virus having various amino acids deleted. Methods of using the vaccine compositions are also included.

Both distinguishable serotypes of Herpes Simplex Virus (HSV-1 and HSV-2) cause infection and disease ranging from relatively minor fever blisters on lips to severe genital infections, and generalized infections of newborns. HSV-1 and HSV-2 are 50% homologous at the DNA level, and polyclonal antibodies and MAbs to shared epitopes are cross-reactive.

HSV-1 and HSV-2 have ribonucleotide reductase 1 (RR1) proteins (an allosteric subunit of the ribonucleotide reductase (RR) enzyme) (respectively designated ICP6 and ICP10) that contain a unique amino terminal domain. The HSV-2 unique domain codes for a serine/threonine-specific protein kinase (PK) which has auto- and transphosphorylating activity and has a transmembrane (TM) domain. Sequences which code for the PK domain cause neoplastic transformation and are associated with cervical cancer (HSV-2 oncogene). The unique terminal domain of the HSV-1 RR1 protein (ICP6) also has PK activity but it is different from that of the HSV-2 oncogene both structurally and functionally.

Original studies, using enzymatic assay conditions similar to those employed for ICP10 PK, concluded that ICP6 does not have PK activity, although the unique domain is retained (Chung, T.D., Wymer, J.P., Kulka, M., Smith, C.C. and Aurelian, L., *Protein kinase activity associated with the large subunit of herpes simplex virus type 2 ribonucleotide reductase (ICP10) Journal of Virology*, Vol. 63, pp.3389-3398, 1989).(Chung '89). This was not unexpected since the sequence of the unique PK domains showed only 38% homology (Nikas, I., McLauchlan, J., Davison, A. J., Taylor, W. R. and Clements, J. B., *Structural features of ribonucleotide reductase. Proteins: Structure, Function and Genetics* 1, pp.376-384, 1986). Further studies indicated that ICP6 has PK activity but only under different

conditions. Also there is controversy as to whether the activity is both auto- and transphosphorylating (see Peng, T., Hunter, J. R. C., and Nelson, J. W. *The novel protein kinase of the RR1 subunit of herpes simplex virus has autophosphorylation and transphosphorylation activity that differs in its ATP requirements for HSV-1 and HSV-2. Virology*, Vol. 216, pp.184-196, 1996 [Peng '96] for a review of the problem; particularly Table 1). The reason for the different PK activities of the ICP6 and ICP10 proteins is likely to be that the ATP binding sites of ICP6 PK are located distantly from the rest of the catalytic motifs (Cooper, J., Marsden, H., and Clements, J. B. *Ribonucleotide reductase of herepesviruses. Journal of Virology*, Vol. 69, pp.4979-4985, 1995). ICP6 also does not have a functional TM domain and it does not localize to the cell surface (Conner, J. Murray, J., Cross, A., Clements, J. B., and Marsden, H. S. *Intracellular localization of herpes simplex virus type 1 ribonucleotide reductase subunits during infection of cultured cells. Virology*, Vol. 213, pp.615, 1995). The PK activity of native ICP6 is very weak even under ideal conditions, such that its K_m is 10-fold higher than that of ICP10 PK (Peng '96).

The transforming activity of ICP6 is located within a genome fragment that is distant from that at which the HSV2 oncogene is located. Transformation in this system is based on focus formation.

It has previously been shown that DNA sequences which encode for the amino-terminal one-third of ICP10 (amino acids 1-411) have oncogenic potential. Cells transfected with these DNA sequences evidence anchorage independent growth and cause tumors in animals. Transformation is seen in both rodent and human cells (Jariwalla, R. J., Aurelian, L. and Ts'o, P.O.P *Tumorigenic transformation induced by a specific fragment of DNA from herpes simplex virus type 2. Proceedings of the National Academy of Sciences*, Vol. 77, pp.2279-2283, 1980 [Jariwalla '80]).

There are three functional domains within ICP10 amino acids 1-411: (i) an intracellular domain at amino acids 106-411 which encompasses the PK catalytic domain with eight conserved catalytic motifs, (ii) a TM at amino acids 88-105 and (iii) an extracellular domain at amino acids 1-88 (Chung '89; Virology, Vol. 179, pp.168-178, 1990). The minimal size required for PK activity is amino acids 1-283 ($pp29^{1a1}$) (Luo, J. H., Smith, C. C., Kulka, M., and Aurelian, L. *A truncated protein kinase domain of the large subunit of herpes simplex virus type 2 ribonucleotide reductase (ICP10) expressed in Escherichia coli. Journal of Biological Chemistry*, Vol. 266, pp. 20976-20983, 1991) [Luo '91]. However, the

PK activity of $pp29^{[a]}$ has some properties different from the authentic ICP10 PK, presumably because it lacks part of PK catalytic domain VI (Luo '91). The TM domain is also required (but insufficient) for PK activity (Luo, J. H. and Aurelian, L. *The transmembrane helical segment but not the invariant lysine is required for the kinase activity of the large subunit of Herpes simplex virus type 2 ribonucleotide reductase. Journal of Biological Chemistry*, Vol. 267, pp.9645-9653, 1992) [Luo '92]. Therefore, it can be concluded that the PK activity is localized within amino acids 88-411 with an essential core at amino acids 88-283.

The unique HpaI site within the ICP10 coding region represents the 3' end of the transforming region (Jariwalla '80) and HpaI cuts the gene after the codon for amino acid residue 417. It is not known whether $pp29^{[a]}$ has transforming activity. However, PK activity is required for neoplastic potential. PK negative mutants do not transform cells. This includes a mutant deleted in the TM domain and site directed mutants in the ATP binding sites (Lys^{176} and/or Lys^{259}) or the ion-binding site (Glu^{209}) (Smith C. C., Luo, J. H., Hunter, J. C. R., Ordonez, J. V., and Aurelian, L. *The transmembrane domain of the large subunit of HSV-2 ribonucleotide reductase (ICP10) is required for protein kinase activity and transformation-related signaling pathways that results in ras activation*. Virology, Vol. 200, pp.598-612, 1994) [Smith '94]. Because a PK⁻ mutant deleted only in the TM domain does not have transforming activity (Smith '94), DNA sequences that code for ICP10 amino acids 106-411 but lack PK activity are not intrinsically neoplastic. This demonstrates that: (i) the HSV-2 oncoprotein is located within ICP10 amino acids 1-411, and (ii) neoplastic potential requires a functional PK activity.

The function of ICP10 PK in virus growth/pathogenesis is unknown.

The HSV-2 ICP10 protein has intrinsic PK activity. This was shown by demonstrating that ICP10 PK activity is lost through site-directed mutagenesis. The oncoprotein also has SH3-binding motifs at positions 140, 149 and 396, which are required for interaction with signaling proteins. This interaction is required for transforming activity. Site directed mutagenesis was used to identify amino acids required for kinase activity and interaction with signaling proteins. Mutation of Lys^{176} or Lys^{259} reduced PK activity (5-8 fold) and binding of the ^{14}C-labeled ATP analog p-fluorosulfonylbenzoyl 5'-adenosine (FSBA), but did not abrogate them. Enzymatic activity and FSBA binding were abrogated by mutation of both Lys residues, suggesting that either one can bind ATP. Mutation of Glu^{209} (PK catalytic motif

III) virtually abrogated kinase activity in the presence of Mg^{2+} or Mn^{2+} ions, suggesting that Glu^{209} functions in ion-dependent PK activity.

ICP10PK functions as a growth factor receptor involved in signaling and it binds the adaptor protein Grb_2 *in vitro*. It has also been shown that there are SH3-binding sites within the ICP10 PK domain (at positions 140, 149 and 396) and they are required for interaction with signaling proteins and, thereby transformation (Nelson , J. W., Zhu, J., Smith, C. C., Kulka, M. and Aurelian, L. *ATP and SH3 binding sites in the protein kinase of the large subunit of herpes simplex virus type 2 of ribonucleotide reductase (ICP10). Journal of Biological Chemistry* 271, pp.17021-17027, 1996) [Nelson '96]. Mutation of the ICP10 proline-rich motifs at position 396 and 149 reduced Grb_2 binding 20- and 2-fold respectively. Binding was abrogated by mutation of both motifs. Grb_2 binding to wild type ICP10 was competed by a peptide for the Grb_2 C-terminal SH3 motif indicating that it involves the Grb_2 C-terminal SH3 (Nelson '96).

The ICP10 PK catalytic domain also contains amino acids that are responsible for binding a down-regulator of PK activity (ras-GAP). They are located at position 106-178. Deletion of these amino acids reduces but does not abrogate PK activity (Luo '92). However, it abrogates ras-GAP binding and thereby increases transforming potential.

The construction of a virus deleted in ICP10 amino acids 106-446 (ICP10ΔPK) is described by Peng '96. Briefly, the wild type sequences in a plasmid (TP101) that contains the HSV-2 BamHI E and T fragments were replaced with the 1.8kb SalI/BglII fragment from pJHL9. pJHL9 is a plasmid containing an ICP10 mutant deleted in the PK catalytic domain '92). The resulting plasmid, TP9, contains sequences which code for ICP10 deleted in the PK catalytic domain flanked by 4 and 2.8 kb of HSV-2 DNA sequences at the 5' and 3' ends, respectively. The 10kb HindIII/EcoRI fragment from TP9 was introduced by marker transfer into a virus (ICP10ΔRR) in which the RR domain of ICP10 had been replaced with the LacZ gene. The resulting recombinant virus, designated ICP10ΔPK, was obtained by selecting white plaques on a background of blue plaques after staining with X-gal. A few white plaques were picked, purified and grown in Vero cells with 10% serum.

The ICP10ΔPK virus is deleted in ICP10 amino acids 106-446. It lacks ICP10 PK activity and retains RR activity (Peng '96) and is attenuated for growth in culture and in infected animals. The virus induces HSV-specific T cell immunity and protects mice from challenge with wild type HSV-2.

There are several known HSV vaccines in the prior art. US patents 4,347,127; 4,452,734; 5,219,567; and 5,171,568 each teach subunit vaccines which provide some protection against HSV-2 infection. These vaccines are inferior to one in which a live, attenuated virus is used. The immunity induced by a subunit vaccine is restricted to the particular protein represented by the subunit, which may not have sufficient protective potential. Additionally it is non-replicating and there is therefore no amplification of the protein which would further reduce immunogenicity. These problems occur in any subunit vaccine regardless of whether the method of preparation is via a recombinant protein or a purification of antigen from a virus.

A cross recombinant vaccine, such as disclosed in US 4,554,159 ('159), does not suffer from the problems of the subunit vaccines, but contains the oncogene present in HSV-2. Unless care is taken to define and delete the oncogene, the cross recombinant vaccine could induce cancer in the vaccinee.

The cross recombinant of '159 is temperature sensitive. Avirulence may be obtained by selecting temperature resistance, but the temperature of the mouse is 39°C while that of humans is 37°C. This temperature sensitivity could well render such a cross problematic in a vaccine. A superior method of selection of avirulence is by the removal of genes coding for virulence without respect to the temperature at which the virus replicates. Also, the use of prototypical crosses would preclude the use of gene deleted or inserted mutants.

Due to the many type-common epitopes on HSV-1 and HSV-2, cell-mediated immunity cross-reacts (Jacobs, R. P., Aurelian, L., and Cole, G. A. *Cell-mediated immune response to herpes simplex virus: Type specific lymphoproliferative responses in lymph nodes draining the site of primary infection. Journal of Immunology*, Vol. 116, pp.1520-1525, 1976).

A live vaccine is superior to a dead vaccine because the live vaccine induces herd immunity; it induces different types of immunity, such as mucosal, cell mediated and humoral immunity. A higher level of immunity is normally obtained because the virus titers are increased through replication within the vaccinee. Finally a live vaccine is of longer duration, thus obviating boosters and lowering initial dosage. The ICP10ΔPK virus is deleted in ICP10 amino acids 106-446. It lacks ICP10 PK activity and retains RR activity (Peng '96) and is attenuated for growth in culture and in infected animals. The virus induces HSV-specific T cell immunity and protects mice from challenge with wild type HSV-2. A patent

application for ICP10ΔPK virus was submitted. However, the levels of HSV-specific immunity induced by ICP10ΔPK virus are 3-fold lower than those induced by similar doses of HSV-2, suggesting that it has a reduced immunogenic potential. An absolute necessity for a live herpes vaccine is the removal of the gene responsible for causing transformation while retaining immunogencity, as in the present invention.

Most developed vaccines (viz. those in neurovirulence genes) are in HSV-1. Known vaccines are not virus type-specific. All known vaccines for HSV-1 or HSV-2 are cross-reactive and provide immunity to the other virus type. However, HSV-1 is not as desirable a vaccine candidate against herpes, because the major clinical problem is the sexually transmitted HSV-2, which is also associated with cancer induction. Recent studies indicate that the age-adjusted prevalence of HSV-2 in the US is now 20.8%, an increase of approximately 30% over the past 13 years (Fleming, D.T., McQuillan, G. M., Johnsons, R. E., Nahmias, A. J., Aral, S. O. Lee, F. K., St Louis, M. E. *Herpes simplex virus type 2 in the United States, 1976 to 1994. New England Journal of Medicine*, Vol. 337, pp.1105-1111, 1997). The increasing rate of HSV-2 acquisition among young adults increases the likelihood that infants will be exposed to HSV-2 at delivery, resulting in an infection that, despite antiviral therapy, is still life-threatening (Whitley, R. J., and Gnann, J. W. Jr. *Acyclovir: a decade later. New England Journal of Medicine*, Vol. 327, pp.782-799, 1992 [Erratum, New England Journal of Medicine, Vol. 328, pp.671, 1993]). A new concern about HSV-2 infection is that it may facilitate the spread of HIV and increase the severity of the disease. Because HSV-1 has only a 50% homology to HSV-2, this may lower the response rate against the heterologous strain in the vaccinated population.

Another absolute requirement for a live vaccine is the absence of lesions upon immunization. An important trait in the live vaccine is its ability to induce high levels of HSV-specific T cell immunity and prevent lesions due to infection with wild type HSV-2 and HSV-1.

A desirable trait in the live vaccine would also be its ability to cause a reduction in the frequency of recurrent lesions in a person already infected. There is a substantial population already infected with HSV who may have intercourse with uninfected individuals who would benefit from such a vaccine.

The present invention solves all the problems recited above providing a whole live attenuated HSV-2 in which the HSV-2 has a deletion of the oncogene, has strong

immunogencity, and may be formulated in a vaccine composition. The present invention provides a method of immunizing a subject against HSV-1 or HSV-2 with said vaccine composition, providing a superior method of conferring immunity upon the subject.

BRIEF SUMMARY OF THE INVENTION

The present invention is a vaccine composition comprising Herpes Simplex Virus-2 recombinant selected from the group of recombinants which have deletions in the gene for ICP10 to remove the fragments encoding for amino acids 107-351 (AuV 351), the fragments encoding for amino acids 107-375 (AuV375) and the fragments encoding for amino acids 107- 417 (AuV417), and a pharmaceutically acceptable carrier or diluent.

It is an object of the present invention to provide a vaccine composition which when administered to an animal, including a human, provides protection from challenge with HSV-2 or HSV-1 infection and may reduce the frequency of recurrent lesions.

It is a further object of the invention to provide a vaccine composition comprising whole, live, attenuated HSV-2 wherein the oncogene or any portion thereof that causes transformation and does not attenuate the virus has been deleted.

It is a further object of the invention to provide a method of immunizing a subject against HSV-2 or HSV-1 comprising administering a novel vaccine composition

It is even a further object of the present invention to reduce or prevent clinical symptoms associated with HSV-2 or HSV-1 infections comprising administering a novel vaccine composition.

It is yet a further object of the present invention to reduce recurrent disease associated with HSV-2 or HSV-1 in previously infected subjects comprising administering a novel vaccine composition.

BRIEF DESCRIPTION OF DRAWINGS

Fig. 1. Schematic representation of the construction of AuV351 DNA.

Fig. 2. Schematic representation of the construction of AuV375 DNA.

Fig. 3. Schematic representation of the construction of AuV417 DNA.

DETAILED DESCRIPTION OF THE INVENTION

In the present invention, live whole HSV-2 has been mutated and attenuated to prevent neoplastic transformation. The mutated HSV-2 can be formulated with immune

stimulants or adjuvants and used to immunize a subject against HSV-1 or HSV-2. The protein kinase (PK) domain of the large subunit of ribonucleotide reductase (ICP10) has previously been shown to have oncogenic properties. Deletion of the PK domain was shown to have deleterious effects on the ability of HSV-2 to infect cells. The present invention consists of the construction of three viruses that have various deletions in the ICP10 PK domain. These deletions encompass the minimal kinase catalytic region but do not include antigenic sites downstream thereof. The mutants are, therefore, PK negative, growth attenuated, and do not have oncogenic potential. Unlike the recombinant virus (ICP10ΔPK) previously constructed (Peng 96), they have superior immunogenicity

Computer assisted analysis of the ICP10 PK antigenicity profile indicates that many antigenic sites are clustered within amino acids 350-450. Because these amino acids are not required for PK activity (Luo '91) the recombinant viruses described in this invention were constructed to retain these antigenic sites. The immunogenicity of amino acids located at this position is also demonstrated by their ability to induce antibody that specifically stains HSV-2 infected, but not uninfected cells. Therefore, viruses that retain these antigenic sites have increased immunogenic potential.

Previous studies documented that HSV-2 RR activity depends on the binding of the two RR subunits (RR1 and RR2) at RR1 sites located within amino acids 419-432 and the extreme C-terminal 145 codons (Chung, T.D., Luo, J. H., Wymer, J.P., Smith, C.C. and Aurelian, L., *Leucine repeats in the large subunit of herpes simplex virus type 2 ribonucleotide reductase (RR:ICP10) are involved in RR activity and subunit complex formation. Journal of General Virology*, Vol. 72, pp.1139-1144, 1991). The significance of these sites for the interaction of the two RR subunits is still controversial, however. For example, working with HSV-1 infected cells, Bonneau, A. M., Kibler, P., White, P., Bousquet, C., Dansereau, N. and Cordingley, M.G. (*Resistance of herpes simplex virus type 1 to peptidomimetic ribonucleotide reductase inhibitors selection and characterization of mutant isolates. Journal of Virology*, Vol. 70, pp.787-93, 1996) concluded that the interaction only requires the C-terminal RR1 amino acids. This interpretation is supported for HSV-2 by the finding that ICP10ΔPK, which is deleted in ICP10 amino acids 106-446, retains RR activity (Peng '96). However, the RR activity of the ICP10ΔPK virus is lower than that of HSV-2. Also oligopeptides that encompass ICP10 amino acids 413-425 and 426-438 reduce HSV-2 RR activity *in vitro* (58 and 31% respectively) (Table 1), presumably because they

cause subunit dissociation. Therefore, recombinant viruses that retain these amino acids, such as those described in this invention, are likely to have a higher RR activity. The increased RR activity may provide an advantage in that the recombinant viruses do not have defects other than the PK which is required for the expression of the regulatory IE genes. As such they will retain attenuated growth mediated by the absence of ICP10 PK while evidencing improved *in vivo* expression required for increased immunogenicity (also favored by the retention of amino acids within positions 350-450).

Therefore, novel vaccine compositions have been discovered and a novel method of immunizing a subject against HSV-2 or HSV-1.

Table 1. HSV-2 RR activity is inhibited by oligopeptides at positions 413-438

Oligopeptide	Inhibition (%)
None	0
aa 165-179	0
aa 355-369	10
aa 413-425	58
aa 426-438	31

RR activity was assayed as described (Averett, D. R., Lubbers, C., Elion, G. B., and Spector, T. *Ribonucleotide reductase induced by herpes simplex virus type 1. Characterization of a distinct enzyme. Journal of Biological Chemistry* 258, pp.9631-9638, 1983) in the absence or presence of 0.5 mM oligopeptides located at various ICP10 amino acids (aa).

HSV-1 and HSV-2 viruses are very similar. The DNA is 50% homologous. Virtually all viral proteins have both type-specific and type common epitopes. For all but 2 proteins (i.e., for 82 proteins), the type-common epitopes are predominant. The exception is the HSV-2 gG2 (Lee, F. K., Coleman, R. M., Pereira, L., Bailey, P.D., Tatsuno, M., and Nahmias, A. J. *Detection of herpes simplex virus type-2-specific antibodies by enzyme-linked immunosorbent*

assay. Journal of Clinical Microbiology. Vol. 22. pp. 641-644. 1985) and the HSV-2 oncoprotein ICP10PK. both of which elicit predominantly type-specific antibodies. In the present invention. the HSV-2 oncogene was deleted from ICP10ΔPK. Therefore we only have one protein that can induce type specific immunity. The remaining 83 proteins will induce type common immunity. This incudes both antibody and cell mediated immunity.

Previously. live whole HSV-2 could not be explored as a vaccine option for HSV since the oncogene had potential neoplastic implications for the patient. The present invention demonstrates that by removing the oncogene. a protein kinase, from the HSV-2 genome, not only are the neoplastic properties removed. but the virus is attenuated and provides full protection against challenge for an extended period of time.

The particular HSV-2 strain which contains the deleted oncogene is not critical to the present invention. Examples of such strains include HSV-2(G). HSV-2(333). HSV-2(186), HSV-2(S-I). although any strain is acceptable. These strains are well known and readily available.

The construction of the mutant virus is accomplished by well known techniques. The location of the oncogene (PK) is well-known (DNA Tumor Viruses Oncogenic Mechanisms, Ed. C. Barbanti-Brodano. et al., Plenum Press. NY. 1995. chapter 14 by L. Aurelian, Transformation and Mutagenic Effects Induced by Herpes Simplex Virus Types 1 and 2, pp. 253-280). The oncogene is located in the ICP10 section of the HSV-2 genome. It has previously been shown that the PK activity and oncogenic activity are located within the gene sequence encoding amino acids 88-411. Briefly, the wild type sequences in a plasmid (TP101) that contains the HSV-2 BamHI E and T fragments are replaced with various fragments from pJHL2 [ICP10 mutant deleted in the PK domain (Luo '92)]. The resulting plasmids contain sequences which code for ICP10 deleted in the PK catalytic domain flanked by 4 and 2.8 kb of HSV-2 DNA sequences at the 5' and 3' ends. respectively. The 10kb HindIII/EcoRI fragment from these plasmids are introduced by marker transfer into a virus (ICP10ΔRR) in which the RR domain of ICP10 had been replaced with the LacZ gene. The resulting recombinant viruses. designated AuV351. AuV375 and AuV 417 are obtained by selecting white plaques on a background of blue plaques after staining with X-gal. A few white plaques are picked and purified.

Southern blot hybridization is used to confirm that the viruses are deleted in the ICP10 PK coding region. The AU26 (CCCCTTCATCATGTTTAAGGA) probe is used. It

recognizes a sequence within the ICP10 RR coding region. The hybridizing bands, seen in the recombinant viruses are smaller than the 7.6kb for wild type HSV-2. The band seen for AuV351 DNA is 6.9kb, that seen for AuV375 DNA is 2.2 kb and that seen for AuV417 DNA is 6.6 kb as compared to 7.6kb for HSV-2 or the restored viruses AuV351(R), AuV375(R) and AuV417(R) DNA.

The recombinant viruses can be differentiated from wild type HSV-2 by DNA analysis and by immunoprecipitation/immunoblotting with antibody to epitopes located at ICP10 amino acids retained by the deleted protein, such as the anti-LA-1 antibody (recognizes ICP10 amino acids 13-26) (Aurelian, et al., Cancer Cells 7, pp.187-191, 1989). The proteins recognized by the antibody are significantly smaller than the 140kDa ICP10 protein. AuV417 is 99 kDa; AuV375 is 104 kDa; and AuV351 is 107 kDa.

The oncogene or any portion thereof may be deleted. By the expression "or any portion thereof" we mean any portion of the oncogene which once deleted results in attenuation of the virus and prevents neoplastic transformation of the cells. Determining if PK activity is absent requires expression of the viral gene and subjecting the result to standard PK assays (Chung '89). There is abundant guidance in the prior art as to the sections of the ICP10 gene which is required for PK activity. Determining viral attenuation requires testing in animals to determine absence of lesion formation. The techniques for accomplishing this are standard and well-known in the art.

The resultant mutant viruses are used in infection experiments and compared to infections with wild-type HSV-2 and the restored viruses. The cells used in infection are not critical to the present invention. Any human or animal cell line which can be infected with wild type HSV-2 may be used in the present invention. Examples of such cell lines include Vero cells, HeLa cells, 293 cells, or MRC5 cells (all available from American Type Culture Collection, Rockville, Maryland). ICP10ΔPK can also be grown in cells that constitutively express ICP10, for example JHLa1. It is titrated by plaque assay on Vero cells with MEM-10% FCS and 0.3% human IgG.

Immunizing a subject indicates the standard interpretation well known in the art. Upon administration with the vaccine composition, neutralizing antibodies and cell-mediated immunity are raised in the subject and said antibodies and cell-mediated immunity confer immunity to the subject.

The present invention teaches immunization of a subject against HSV-2. A"pfu" is a plaque forming unit and represents the quantity of virus required to form a single plaque when a cell culture is infected with the virus. It is a quantitative measure of viral infectivity used by those skilled in the art. Due to the 50% homology of HSV-1 and HSV-2 there will be a high degree of protection against HSV-1 infection.

The formulation of viruses AuV351, AuV375 and AuV417 for human use is accomplished by suspension in a solution with or without stabilizing ingredients, and with or without immune stimulants and adjuvants. Examples of stabilizing agents, immune stimulants, and adjuvants include alum, incomplete Freud's adjuvant, MR-59 (Chiron, Emeryville, CA), MPL (mono-phosphoryl Lipid A). Such stabilizing agents, adjuvants and immune stimulants are well known in the art and can be used singly or in combination.

The vaccine composition of the present invention can be administered to any animal, including humans. The vaccine composition may be administered via any suitable mode of administration, such as intramuscular, oral, subcutaneous, intradermal, intravaginal, rectal, or intranasal administration. A preferred mode of administration is subcutaneous or intradermal administration.

The AuV351, AuV375 and AuV417 viruses, which provide protection against HSV-2 infection, can be administered along with a pharmaceutically acceptable carrier or diluent. Examples of such pharmaceutically acceptable carrier or diluents include water, phosphate buffered saline or sodium bicarbonate buffer. A number of other acceptable carriers or diluents are known.

The following examples are provided for illustrative purposes only and are in no way intended to limit the scope of the present invention.

Example 1

Construction and Characterization of the AuV351 virus and AuV351(R)

The strategy for construction of AuV351 is to create a recombinant plasmid, pAuΔ351 that contains a gene cassette deleted in ICP10 amino acids 107-351 (Fig. 1). This plasmid is used for the generation of a recombinant HSV-2 virus deleted in ICP10 amino acids 107-351 through recombination with the appropriate viral DNA. All details of cloning methodology are based on standard procedures.

Plasmid pJL2 (Luo '92) is digested with BamHI (made blunt ended) and StuI to remove the 732bp fragment that encodes amino acids 107-351. The resulting construct is

collapsed through ligation at the BamHI/StuI sites to generate plasmid pΔ351. The wild type sequences in a plasmid (TP101) that contains the HSV-2 BamHI E and T fragments (Peng '96) are replaced with the 2.4kb SalI/BglII fragment from pΔ351. The resulting plasmid, pAuΔ351, contains sequences which code for ICP10 deleted in amino acids 107-351 flanked by 4 and 2.8kb of HSV-2 DNA sequences at the 5' and 3' ends, respectively. The 10.4kb HindIII/EcoRI fragment from pAuΔ351 is introduced by marker transfer into a virus (ICP10ΔRR) in which the RR domain of ICP10 has been replaced with the LacZ gene. The resulting recombinant designated AuV351, is obtained by selecting white plaques on a background of blue plaques after staining with X-gal. A few white plaques are picked, purified, and grown in Vero cells in MEM with 10% FCS. For the construction of the restored virus AuV351(R), Vero cells are co-transfected with 1μg of infectious viral DNA from AuV351 and a 10-fold molar excess of the wild type BamHI E/T fragment. A strategy similar to that reported for ICP6Δ (Goldstein, D. J. and Weller, S. K. *Factor(s) present in the herpes simplex virus type-1 infected cells can compensate for the loss of the large subunit of the viral ribonucleotide reductase: characterization of an ICP6 deletion mutant. Virology*, Vol. 166, pp.41-51, 1988) is used to select restored virus under growth restricted conditions with 1% FCS.

Southern blot hybridization is used to confirm that the AuV351 DNA is deleted in nucleotides encoding ICP10 amino acids 107-351. Viral DNA is isolated from cytoplasmic virions as described (Pignatti et al., Virology, Vol. 93, pp.260-264, 1979; Smith et al., Journal of General Virology, Vol. 73, pp.1417-1428, 1992). Briefly, Vero cells are infected at a multiplicity of infection (moi) of 5. At 48 hrs. post infection (p.i.) cells are resuspended (2 x 10^7 cell/ml) in a buffer consisting of 10 mM Tris-HCl (pH 7.9), 10 mM EDTA and 0.25% Triton. Following incubation on ice (15 min.), NaCl is added at a final concentration of 0.2 M and the nuclei are precipitated by centrifugation at 1,000 x g (10 min, 4°C). The supernatant, containing cytoplasmic virions, is incubated in 200 μg/ml Proteinase K and 0.2% SDS (4 hr at 37°C), mixed with saturated sodium iodide (NaI; final concentration 1.525 g/ml) and ethidium bromide (final concentration 3 μg/ml) and centrifuged at 100,000xg for 16 hrs.

Viral DNA (15 μg) is digested with BamH I and the fragments are separated by 1% agarose gel electrophoresis in a Tris-Acetate EDTA (TAE) buffer (40 mM Tris-acetate and 1 mM EDTA). It is transferred to Gene screen membranes (New England Nuclear Corp., Beverly, MA) and the membranes are incubated in a prehybridization solution containing 5 x

SSC [750 mM NaCl. 75 mM Sodium citrate: pH (7.0)], 2% Casein. 0.1% N-laurylsarcosine and 0.02% sodium dodecyl sulfate (SDS)] at 42° C for 2 hrs. The hybridization probe is oligonucleotide AU26 (CCCCTTCATCATGTTTAAGGA) which represents a sequence in the ICP10 RR coding region. It is 3' tailed with digoxigenin-dUTP (DIG-dUTP) by terminal transferase (Boehringer Mannheim, Indianapolis, IN) in 20 µl volume with lx reaction buffer [5 mM cobalt chloride ($CoCl_2$), 0.05 mM DIG-dUTP, 5 nmol/ml AU26, 0.5 mM dATP and 2.5 units/µl terminal transferase] at 37°C for 15 min. diluted to a final concentration of 5 pmol/ml in prehybridization solution. Hybridization is done at 42°C for 3 hrs. Membranes are washed once (room temperature) in a solution containing 2xSSC, 0.1% SDS for 5 mins and twice in 0.5xSSC, 0.1% SDS for 15 mins. For detection of the hybridized DNA fragments, the membranes are rinsed in Buffer 1(100 mM Tris-HCl, pH 7.5,150 mM NaCl), incubated in Buffer 2 [2% (w/v) casein in Buffer 1] for 40min and in Buffer 2 containing $3x10^{-4}$U/ml of alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim, Indianapolis, IN) for 30 min. After washing with Buffer 1 (twice) and soaking in Buffer 3 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$ for 2 min, the membranes are exposed to the chemiluminescent substrate Lumi-Phos™ 530 (Boehringer Mannheim, Indianapolis, IN) and the reaction is developed on X-ray film.

More specifically, DNA (15 µg) from HSV-2, AuV351 or AuV351(R) is digested with BamHI, separated on 1% agarose gels and transferred to nylon membranes. It is hybridized with the AU26 probe which recognizes a sequence within the ICP10 RR coding region. A hybridizing 7.6kb band which represents the BamHI E fragment is observed for HSV-2, and AuV351(R) DNA. The hybridizing band seen for AuV351 DNA is 6.9kb.

Example 2

Construction and Characterization of the AuV375 virus and AuV375(R)

The strategy for construction of AuV375 is to create a recombinant plasmid, pAuΔ375 that contains a gene cassette deleted in ICP10 amino acids 107-375 (Fig. 1). This plasmid is used for the generation of a recombinant HSV-2 virus deleted in ICP10 amino acids 107-375 through recombination with the appropriate viral DNA. All details of cloning methodology are based on standard procedures.

Plasmid pJL2 (Luo '92) is digested with EcoNI (partial) and BamHI to remove the 804bp fragment that encodes amino acids 107-375. After both sites are made blunt-ended, the resulting construct is collapsed through ligation at the BamHI/EcoNI sites to generate

plasmid pΔ375. The wild type sequences in a plasmid (TP101) that contains the HSV-2 BamHI E and T fragments (Peng '96) are replaced with the 2.3kb SalI/BglII fragment from pΔ375. The resulting plasmid, pAuΔ375, contains sequences which code for ICP10 deleted in amino acids 107-375 flanked by 4 and 2.8kb of HSV-2 DNA sequences at the 5' and 3' ends, respectively. The 10.3kb HindIII/EcoRI fragment from pAuΔ375 is introduced by marker transfer into a virus (ICP10ΔRR) in which the RR domain of ICP10 has been replaced with the LacZ gene. The resulting recombinant, designated AuV375, is obtained by selecting white plaques on a background of blue plaques after staining with X-gal. A few white plaques are picked, purified, and grown in Vero cells in MEM with 10% FCS. For the construction of the restored virus AuV375(R), Vero cells are co-transfected with 1μg of infectious viral DNA from AuV375 and a 10-fold molar excess of the wild type BamHI E/T fragment. A strategy similar to that reported for ICP6Δ (Goldstein and Weller, Virology, Vol.166, pp.41-51, 1988) is used to select restored virus under growth restricted conditions (1 % FCS).

Southern blot hybridization is used to confirm that the AuV375 DNA is deleted in nucleotides encoding ICP10 amino acids 107-375 as described in Example 1.

More specifically, DNA (15 μg) from HSV-2, AuV375 or AuV375(R) is digested with BamHI, separated on 1% agarose gels and transferred to nylon membranes. It is hybridized with the AU26 probe which recognizes a sequence within the ICP10 RR coding region. A hybridizing 7.6kb band which represents the BamHI E fragment is observed for HSV-2, and AuV375(R) DNA. The hybridizing band seen for AuV375 DNA is 2.2kb.

Example 3

Construction and Characterization of the AuV417 virus and AuV417(R)

The strategy for construction of AuV417 is to create a recombinant plasmid, pAuΔ417 that contains a gene casette deleted in ICP10 amino acids 107-417 (Fig. 3). This plasmid is used for the generation of a recombinant HSV-2 virus deleted in ICP10 amino acids 107-417 through recombination with the appropriate viral DNA. All details of cloning methodology are based on standard procedures.

Plasmid pJL2 (Luo '92) is digested with BamHI (made blunt-ended) and HpaI to remove the 936bp fragment that encodes amino acids 107-417. The resulting construct is collapsed through ligation at the BamHI/EcoNI sites to generate plasmid pΔ417. The wild type sequences in a plasmid (TP101) that contains the HSV-2 BamHI E and T fragments

(Peng '96) are replaced with the 2.1kb SalI/BglII fragment from pΔ417. The resulting plasmid, pAuΔ417, contains sequences which code for ICP10 deleted in amino acids 107-417 flanked by 4 and 2.8kb of HSV-2 DNA sequences at the 5' and 3' ends, respectively. The 10.1kb HindIII/EcoRI fragment from pAuΔ417 is introduced by marker transfer into a virus (ICP10ΔRR) in which the RR domain of ICP10 has been replaced with the LacZ gene. The resulting recombinant designated AuV417, is obtained by selecting white plaques on a background of blue plaques after staining with X-gal. A few white plaques are picked, purified, and grown in Vero cells in MEM with 10% FCS. For the construction of the restored virus AuV417(R), Vero cells are co-transfected with 1μg of infectious viral DNA from AuV417 and a 10-fold molar excess of the wild type BamHI E/T fragment. A strategy similar to that reported for ICP6Δ (Goldstein and Weller, Virology, Vol. 166, pp.41-51, 1988) is used to select restored virus under growth restricted conditions (1% FCS).

Southern blot hybridization is used to confirm that the AuV417 DNA is deleted in nucleotides encoding ICP10 amino acids 107-417 as described in Example 1.

More specifically, DNA (15 μg) from HSV-2, AuV417 or AuV417(R) is digested with BamHI, separated on 1% agarose gels and transferred to nylon membranes. It is hybridized with the AU26 probe which recognizes a sequence within the ICP10 RR coding region. A hybridizing 7.6kb band which represents the BamHI E fragment is observed for HSV-2, and AuV417(R) DNA. The hybridizing band seen for AuV417 DNA is 6.6kb.

Example 4

AuV351, AuV375 and AuV417 have higher RR activity than ICP10ΔPK

ICP10ΔPK virus has RR activity, but it is lower than that of HSV-2. Inasmuch as amino acids 413-438 may be involved in the complexation of the two RR subunits (Table 1), viruses that retain these amino acids have an RR activity similar to that of HSV-2 (Table 2).

are isolated from the footpad and ganglionic homogenates for 7-9 days. AuV351, AuV375 and AuV417 are isolated for only 3-4 days. Maximum titers and the proportion of latently infected ganglia are lower than those seen for HSV-2 (1-3x10^7 pfu and 80-90% latency).

Example 6

AuV351, AuV375 and AuV417 protect from HSV-2 challenge.

The footpad model described in example 5 is used to examine protection by AuV351, AuV375 and AuV417. The experiment is done as previously described (Wachsman '89; Wachsman '92) with mice given one or multiple immunizations with 1x10^7 pfu of virus (at 14-16 days intervals) before challenge with wild type HSV-2. Challenge is with 1x10^7 to 1x10^8 pfu of HSV-2 and it is done at 3-6 weeks after the last immunization. All mice in the PBS group develop skin lesions from which virus is isolated, and 50-80% die on days 8-13 after challenge. By contrast, lesions are not seen and virus is not isolated from the immunized mice. The AuV351, AuV375 and AuV417 viruses have vaccine potential.

Example 7

AuV351, AuV375 and AuV417 viruses induce HSV-specific immunity.

Groups of mice are immunized with AuV351, AuV375 and AuV417 as described in example 6. Two-four weeks after the last injection spleens are removed and T cells are used in lymphocyte proliferation assays as described (Wachsman *et al.*, Journal of General Virology, Vol. 70, pp.2513-2520, 1989; Vaccine, Vol. 10, pp.447-454, 1992). HSV-specific lymphoproliferation is seen in all animals. Proliferative levels are similar to those seen for HSV-2. AuV351, AuV375 and AuV417 induce good levels of virus-specific T cell responses. They also induce antibody responses as determined by neutralization assays.

All references cited herein are incorporated by reference in their entirety.

It will be apparent to those skilled in the art that the examples and embodiments described herein are by way of illustration and not of limitation, and that other examples may be utilized without departing from the spirit and scope of the present invention, as set forth in the appended claims.

Table 2. Ribonucleotide reductase activity of ICP10ΔPK virus.

Virus	RR Specific activity (Units)[a]
HSV-2	10.2
ICP10ΔPK	8.0
AuV351	10.2
AuV375	10.2
AuV417	10.2
Mock-infected	2.7

[a] One RR unit = conversion of 1 nmol CDP to dCDP/h/mg protein. Data are experimentally determined for HSV-2, ICP10ΔPK and Mock-infected. They are projected for AuV351, AuV375 and AuV417

Example 5

AuV351, AuV375 and AuV417 are attenuated for growth in infected animals.

The mouse footpad model of HSV-2 infection is used to examine the growth of AuV351, AuV375 and AuV417 *in vivo*. Seven groups of Swiss Webster mice are inoculated s.c. in the footpad with $1x10^7$ pfu of HSV-2, AuV351, AuV375, AuV417 or the restored viruses AuV351(R), AuV375(R) and AuV417(R). Neurological symptoms and severe skin lesions are seen in mice given HSV-2 or the restored viruses beginning on day 6 p.i. (Wachsman, M. Luo, J. H., Aurelian, L., Perkus, M. E., and Paoletti, E. *Antigen-presenting capacity of epidermal cells infected with vaccinia virus recombinants containing the herpes simplex virus glycoprotein D and protective immunity. Journal of General Virology*, Vol. 70, pp.2513-2520, 1989 [Wachsman '89]; (Wachsman, M. Luo, J. H., Aurelian, L., and Paoletti, E. *Protection from herpes simplex virus type 2 is associated with T cells involved in delayed type hypersensitivity that recognize glycosylation-related epitopes on glycoprotein D. Vaccine*, Vol. 10, pp.447-454, 1992) [Wachsman '92]. Mice infected with AuV351, AuV375 or AuV417 have no neurological symptoms nor skin lesions. HSV-2 and the restored viruses

CLAIMS

We claim:

1. A vaccine composition comprising a live Herpes Simplex Virus Type-2 recombinant virus having a genome with a deletion in the gene coding for the protein kinase domain of the large subunit of ribonucleotide reductase and a pharmaceutically acceptable carrier or diluent.

2. The vaccine composition of claim 1 wherein the live Herpes Simplex Virus-2 recombinant virus is selected from the group consisting of AuV 351, AuV375 and AuV417.

3. A method of immunizing a subject against Herpes Simplex virus comprising the step administering the vaccine composition of claim 1 to said subject.

4. The method of claim 3 wherein said subject is a human.

5. The method of claim 3 wherein the dosage range for said vaccine composition is 1 pfu to 100 million pfu.

6. The method of claim 3 wherein said vaccine composition is administered via an intranasal, oral, intravaginal, subcutaneous or intradermal route.

7. A method of conferring immunity against Herpes Simplex virus to a subject comprising the step administering the vaccine composition of claim 1.

8. The method of claim 7 wherein said subject is a human.

9. The method of claim 7 wherein the dosage range for said vaccine composition is 1 pfu to 100 million pfu.

10. The method of claim 7 wherein said vaccine composition is administered via an intranasal, oral, intravaginal, subcutaneous or intradermal route.

11. A method of preventing clinical symptoms in a subject associated with Herpes Simplex Virus in a subject comprising the step administering the vaccine composition of claim 1 to said subject.

12. The method of claim 11 wherein said subject is a human.

13. The method of claim 11 wherein the dosage range for said vaccine composition is 1 pfu to 100 million pfu.

14. The method of claim 11 wherein said vaccine composition is administered via an intranasal, oral, intravaginal, subcutaneous or intradermal route.

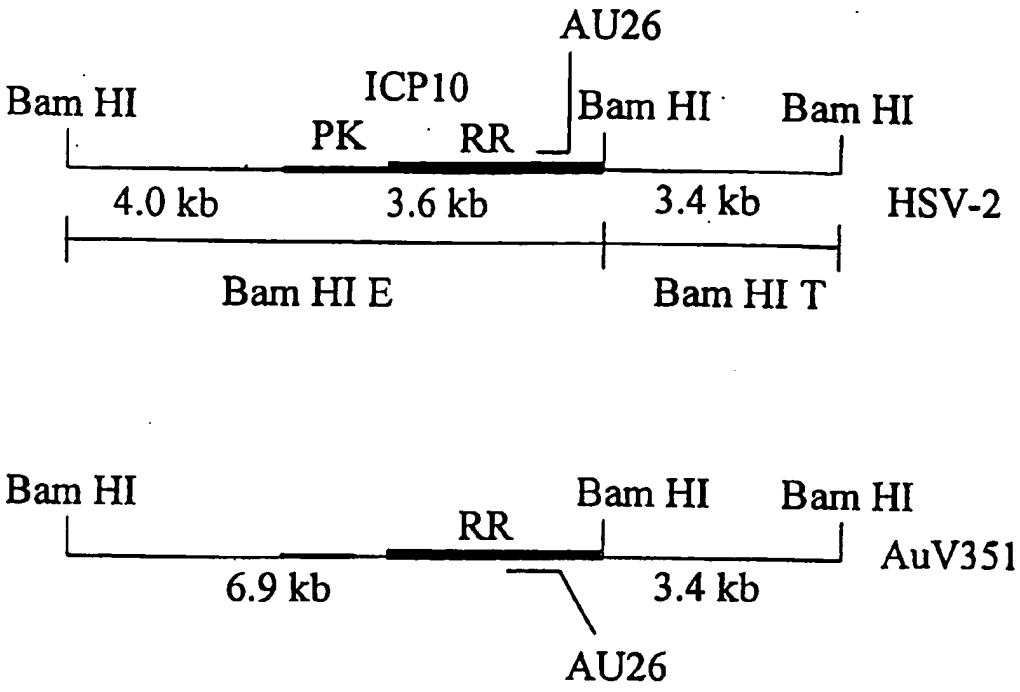
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Fig. 1

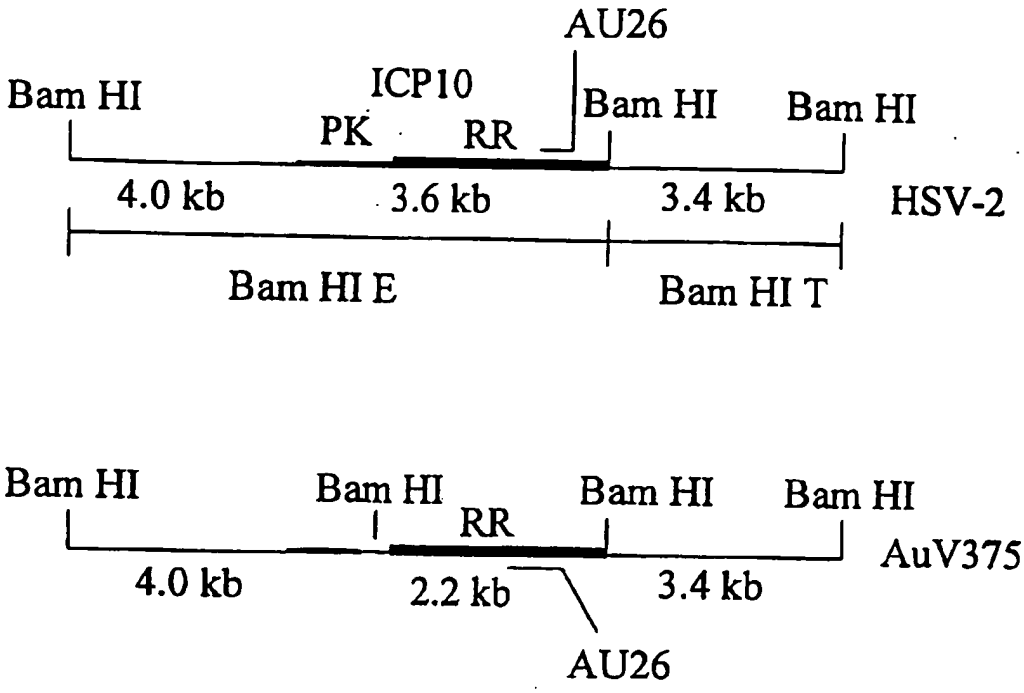
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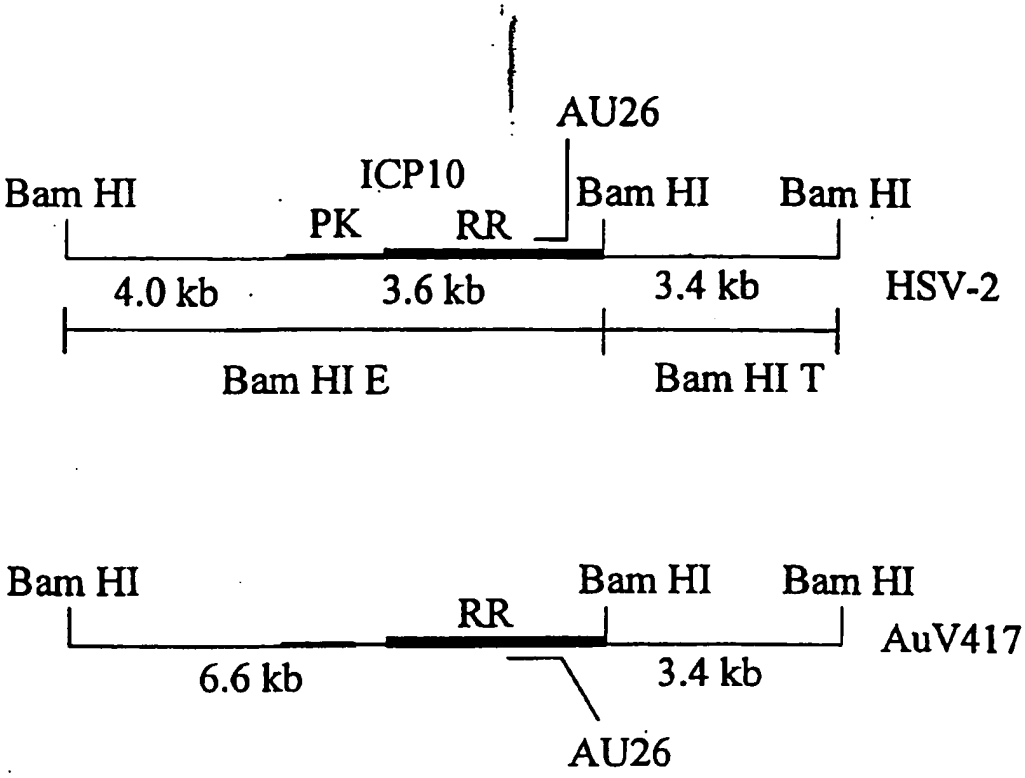
Fig. 2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Bam HI
RR
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3.4 kb
AU26

Fig. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/00922

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 39/12, 39/245; C12N 15/00

US CL : 424/184.1, 199.1, 204.1, 229.1, 231.1; 435/320.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/184.1, 199.1, 204.1, 229.1, 231.1; 435/320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, CAS ONLINE
search terms: Herpes Simplex, Vaccine, protin Kinase, ICP10

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	MILLIGAN et al. Generation of Humoral Immune Responses against Herpes Simplex Virus Type 2 in Murine Female Genital Tract. Virology. 1995, Vol. 206, pages 234-241, see the entire document.	1, 2, 5, 6, 8-10 ------ 3, 4, 7, 11-14
X - Y	PARR et al. A Mouse Model for Studies of Mucosal Immunity to Vaginal Infection by Herpes Simplex Virus Type 2. Laboratory Investigation. 1994, Vol. 70, No. 3, pages 369-380, see the entire document.	1, 2, 5-10 ------- 3, 4, 11-14
A	MURTHY et al. Deletion Mutants of Herpesvirus Saimiri Define an Open Reading Frame Necessary for Transformation. Journal of Virology. August 1989, Vol. 63, No. 8, pages 3307-3314.	1-14

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
05 APRIL 1999

Date of mailing of the international search report
03 MAY 1999

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/00922

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	AURELIAN et al. Amino-terminal Epitope of Herpes Simplex Virus Type 2 ICP10 Protein as a Molecular Diagnostic Marker for Cervical Intraepithelial Neoplasia. Cancer Cells 7. 1989, pages 187-191.	1-14
A	NELSON et al. ATP and SH3 Binding Sites in the Protein Kinase of the Large Subunit of Herpes Simplex Virus Type 2 of Ribonucleotide Reductase (ICP10). The Journal of Biological Chemistry. 19 July 1996, Vol. 271, No. 29, pages 17021-17027.	1-14